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Research

Homoeologous crossovers are distally biased and underlie genomic instability in first-generation neo-allopolyploid *Arabidopsis suecica*

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Summary

- First-generation polyploids often suffer from more meiotic errors and lower fertility than established wild polyploid populations. One such example is the allopolyploid model species *Arabidopsis suecica* which originated *c.* 16 000 generations ago.
- We present here a comparison of meiosis and its outcomes in naturally evolved and first-generation 'synthetic' *A. suecica* using a combination of cytological and genomic approaches.
- We show that while meiosis in natural lines is largely diploid-like, synthetic lines have high levels of meiotic errors including incomplete synapsis and nonhomologous crossover formation. Whole-genome re-sequencing of progeny revealed 20-fold higher levels of homoeologous exchange and eightfold higher aneuploidy originating from synthetic parents. Homoeologous exchanges showed a strong distal bias and occurred predominantly in genes, regularly generating novel protein variants. We also observed that homoeologous exchanges can generate megabase scale INDELs when occurring in regions of inverted synteny. Finally, we observed evidence of sex-specific differences in adaptation to polyploidy with higher success in reciprocal crosses to natural lines when synthetic plants were used as the female parent.
- Our results directly link cytological phenotypes in *A. suecica* with their genomic outcomes, demonstrating that homoeologous crossovers underlie genomic instability in neo-allopolyploids and are more distally biased than homologous crossovers.

Introduction

Polyploidy, also referred to as whole-genome duplication (WGD), is a recurrent event in the evolution of plant genomes (Van de Peer *et al.*, 2009; Jiao *et al.*, 2011; Soltis *et al.*, 2015), and increasingly recognised as an important evolutionary driver outside the plant kingdom (e.g. Donne *et al.*, 2020; Novikova *et al.*, 2020). In plants, the question is not really whether a plant is polyploid, but rather how long has it been since a WGD event last occurred? All angiosperms have undergone at least two rounds of WGD (Jiao *et al.*, 2011), with *c.* 25–30% having a polyploidy event in their recent evolutionary past (Wood *et al.*, 2009; Barker *et al.*, 2016). While polyploidy is a recurrent theme in plant evolution, the establishment of new polyploid species poses many challenges, requiring adaptation to new cellular (Müntzing, 1936), physiological (Chao *et al.*, 2013) and transcriptional (Song *et al.*, 2020) environments. Perhaps the most immediate hurdle to overcome, however, is that of sexual reproduction (Grandont *et al.*, 2013).

Sexual reproduction requires the accurate segregation of homologous chromosome pairs during the first meiotic division. To facilitate this segregation in most species, each chromosome is physically linked with its homologous partner via a meiotic crossover to form a bivalent. The metaphase I spindle assembly checkpoint then ensures the two chromosomes in each bivalent are being pulled towards opposing poles before the cell cycle can progress (Wassmann *et al.*, 2003). In diploids, each chromosome has one homolog thus the choice of partner with which to recombine is a relatively simple one that is the only other chromosome that shares significant homology. In polyploids, partner choice is more difficult. Each chromosome has multiple other homologous chromosomes (autopolyploids) or closely related homoeologous chromosomes (allopolyploids) with which it is possible to pair and recombine. For first-generation polyploids, this choice overload regularly proves too much, and as a result, they tend to have low fertility due to chromosome segregation errors and genomic rearrangements (Darlington, 1929; McCollum, 1958; Szadkowski *et al.*, 2010; Grandont *et al.*, 2013; Yant *et al.*, 2013;

Lloyd & Bomblies, 2016). Such is the challenge faced, that many new polyploids avoid meiosis altogether and only reproduce vegetatively or via apomixis (Herben *et al.*, 2017; Hojsgaard & Hörandl, 2019). However, despite the challenge, many polyploids have navigated this transition and adapted their meiotic programme to enable faithful chromosome segregation and fertility, including the recently formed allopolyploid model *Arabidopsis suecica* (Henry *et al.*, 2014; Burns *et al.*, 2021; Nibau *et al.*, 2022).

In allopolyploids, crossover formation must occur only between true homologues and not between closely related homoeologs. In first-generation allopolyploids, partner choice is often insufficiently stringent and so crossovers are formed between homoeologous chromosomes (Szadkowski *et al.*, 2010; Henry *et al.*, 2014; Zhang *et al.*, 2020), resulting in increased multivalents and univalents, and an associated loss of fertility. Exchanges induced by these crossovers between homoeologs also alter genome structure (Szadkowski *et al.*, 2010) and gene expression patterns (Lloyd *et al.*, 2018) affecting the fertility and viability of subsequent generations. There is clearly a strong selective pressure therefore for new allopolyploids to increase the stringency of partner choice and suppress homoeologous crossovers. Unsurprisingly, loci affecting allopolyploid meiotic stability have been reported in many systems including wheat (Riley *et al.*, 1960), oats (Gauthier & McGinnis, 1968; Rajhathy & Thomas, 1972), *Brassica napus* (Jenczewski *et al.*, 2003), ryegrass (Taylor & Evans, 1977) and *A. suecica* (Henry *et al.*, 2014). Only in wheat, however, have causal genes been identified, with *Ph1* thought to be a nonsyntenic duplicate of *ZIP4* located on Chromosome 5B (Rey *et al.*, 2017) and *Ph2*, a copy of the mismatch repair gene *MSH7* found on chromosome 3D (Serra *et al.*, 2021).

The allopolyploid model *A. suecica* is an attractive system to further elucidate the molecular control of the stringency of meiotic chromosome pairing and crossover formation. *A. suecica* ($2n = 2\times = 26$) is thought to have formed *c.* 16 000 generations ago from the parental species *A. thaliana* ($2n = 2\times = 10$) and *A. arenosa* which is found in both diploid ($2n = 2\times = 16$) and autotetraploid ($2n = 4\times = 32$) cytotypes (O'Kane *et al.*, 1996; Burns *et al.*, 2021; Jiang *et al.*, 2021). While a tetraploid origin for the *A. arenosa* subgenome of *A. suecica* has been suggested (Novikova *et al.*, 2016, 2017), we observed recently that *A. suecica* has diploid variants of key meiotic genes that are highly differentiated between diploid and tetraploid *A. arenosa* (Nibau *et al.*, 2022). As such, the exact nature of the *A. arenosa* subgenome donor remains somewhat unclear. To provide a foundation for future research, we recently characterised meiosis in a common laboratory strain of *A. suecica*, demonstrating that it is largely diploid-like, with no evidence of crossover formation or synapsis between nonhomologous chromosomes, although some abnormalities were seen at low frequency, including univalents at metaphase I (Nibau *et al.*, 2022). By contrast, first-generation synthetic *A. suecica* has low levels of fertility which is assumed to result from errors during meiosis, in particular, incorrect partner choice in prophase I leading to homoeologous recombination and subsequent genomic rearrangements (Henry *et al.*, 2014). There has been some attempt to identify genes that may have evolved to stabilise the meiotic behaviour of *A. suecica*. Using an F2 population derived from a cross between natural and synthetic *A. suecica*, Henry *et al.* (2014) identified a QTL on chromosome nine influencing pollen viability. Plants homozygous for the synthetic allele at this position had lower pollen viability than plants homozygous for the natural allele, as would be expected if this QTL had evolved to regularise the meiotic behaviour of *A. suecica*; however, no candidate meiosis genes were identified underlying the QTL.

Given the interest in the species as a model for allopolyploid meiosis research, we have undertaken a systematic comparison of meiotic behaviour in natural and synthetic *A. suecica*. We use a combination of cytological and genomic approaches to demonstrate that low fertility in *A. suecica* is associated with wide-scale meiotic abnormalities including asynapsis, reduced crossover formation, homoeologous recombination and nondisjunction, resulting in frequent genomic rearrangements in progeny of synthetic plants.

Materials and Methods

Plant materials

Arabidopsis suecica Fr. ecotypes used were AsS3 (Burns *et al.*, 2021), originally obtained from the Magnus Nordborg Lab, and JC2 (Nibau *et al.*, 2022). The *A. arenosa* L. ecotype used was the reference strain SNO (Hollister *et al.*, 2012; Burns *et al.*, 2021), obtained from Kirsten Bomblies (ETH, Zurich). Synthetic *A. suecica* was generated from diploid *A. arenosa* (SNO) and diploid *A. thaliana* L. (Col-0) through colchicine doubling of the diploids (to be described later) and then crossing the synthetic autotetraploids using *A. thaliana* as the maternal parent.

Plants were initially grown in long-day conditions (16 h : 8 h, light : dark cycle) in growth chambers or glasshouses at 21°C. After 4 wk, plants were moved to long-day conditions at 4°C for a minimum of 4 wk for vernalisation before returning to growth chambers at 21°C to induce flowering. Chromosome numbers of all plants were validated before using for any cytological assays to ensure they were euploid (i.e. $2n = 26$).

Pollen viability

Inflorescences were fixed in 3 : 1, ethanol:glacial acetic acid for 48 h with several changes in fixative. Mature anthers, just before dehiscence, were dissected and pollen released in 10 µl Alexander's stain (Peterson *et al.*, 2010) and viability scored using a compound microscope.

DAPI spreads

DAPI spreads were undertaken as described in Chelysheva *et al.* (2010) with minor modifications as described in Nibau *et al.* (2022). Briefly, inflorescences were fixed for a minimum of 3 d in Carnoy's fixative (3 : 1 ethanol : acetic acid, v/v), with several changes of fixative in the first 24 h. Fixed inflorescences

were rinsed in water (once) and 10 mM citrate buffer, pH 4.5 (twice), then incubated in digest mix (0.3% w/v cellulase RS, 0.3% w/v pectolyase Y23 and 0.3% w/v cytohelicase in citrate buffer) at 37°C for 2.5–3.0 h. Digested buds were tapped with a brass rod to transform into a cell suspension on a microscope slide, 12.5 µl of 80% acetic acid added, and stirred continuously with a hook for 2 min on a 45°C heat block with addition of an additional 12.5 µl of 80% acetic acid after 1 min. Cells were fixed on slides by pipetting Carnoy's fixative around the drop of cleared cell suspension and air-dried before mounting in 7 µl of VECTASHIELD (Vector Laboratories, Newark, CA, USA, https://vectorlabs.com) with 2 µg ml^{-1} DAPI.

Fluorescence *in situ* hybridisation

Fluorescence *in situ* hybridisation (FISH) probes specific to the centromeric repeats of the two subgenomes were labelled by polymerase chain reaction with Rhodamine-5-dUTP (*A. thaliana*) or Fluorescein-12-dUTP (*A. arenosa*) and hybridised to meiotic chromosome spreads as described previously (Nibau *et al.*, 2022), before imaging with an epifluorescent microscope.

3D nuclei embedding and immunolabelling

Meiocytes from *A. suecica* (JC2) were embedded in acrylamide to preserve their three-dimensional structure and used for the immunolocalisation studies as described previously (Phillips *et al.*, 2010; Nibau *et al.*, 2020). Briefly, fresh young buds (< 1 mm) were harvested and fixed in 2% (w/v) paraformaldehyde, washed, macerated with a brass rod in 1% (v/v) Lipsol in buffer A and embedded in acrylamide. Embedded meiocytes were blocked and incubated with α-ASY1 (rat, 1 : 500; Armstrong *et al.*, 2002), and α-ZYP1 (guinea pig, 1 : 500; Higgins *et al.*, 2005) antibody solution for 24 to 36 h at 4°C. After washing, embedded meiocytes were incubated overnight at 4°C with secondary antibodies (Alexa Flour 568 goat anti-rat (A11077) and Alexa Fluor 633 goat anti-guinea pig (A21105), Invitrogen) used at 1 : 500 dilution. Images were acquired using a Leica TCS SP8 confocal microscope as maximum projection of *Z*-stacks and deconvolved using the built-in LIGHTNING software (Leica Microsystems UK, Milton Keynes, UK). Image analysis was carried out in IMARIS v.7.3 (Bitplane; Oxford Instruments, Abingdon, UK).

Sequencing

For whole-genome re-sequencing, DNA was extracted from leaf tissue according to a CTAB protocol (Doyle & Doyle, 1987) with minor alterations as described in Nibau *et al.* (2022). DNA was quantified using Qubit dsDNA HS dye (Q32854) from Thermo Fisher Scientific (Life Technologies); fluorescence was measured in black 384-well plates (Greiner Bio-One, Kremsmünster, Austria) using a FLUOstar Omega plate reader (BMG Labtech, Aylesbury, UK) with 485 and 520 nm excitation and emission filters, respectively, with fluorescence intensity compared with that of a known standard. Sequencing libraries were prepared using the LITE library preparation method described by Rowan *et al.* (2019), and sequenced on a single lane of a NovaSeq X (Earlham Institute). Short-read data are available on the NCBI Sequence Read Archive under Bioproject PRJNA1103975.

Sequencing analysis

Our data-processing pipeline involved four main parts: (1) preparing the raw sequencing data; (2) mapping data; (3) variant discovery (GATK 4.2.4.1, following GATK best practices); and (4) copy number variation (CNV) analysis (Orlandini *et al.*, 2017). Fastq.gz files were mapped to the AsS3 *A. suecica* reference genome (GCA_905175345.1) using BWA-MEM 2. We removed duplicate reads using 'MarkDuplicates' from PICARD tools (GATK 4.2.4.1), followed by 'AddOrReplaceReadGroups' to add read groups and indices to the .bam files. We called variants for the .bam files using 'HaplotypeCaller' and 'GenotypeGVCFs' (GATK 4.2.4.1), combining the single-sample GVCF outputs from HaplotypeCaller to a multisample GVCF before running 'GenotypeGVCFs'. Using 'SelectVariants' in GATK, we restricted to biallelic sites and removed sites that had excess read depth, defined as > 2× modal read depth (DP > 1330), and we also applied GATK 'best practice' hard-filtering for SNPs ('DP > 8 && QD > 2.0 && MQ > 40.0 && FS < 60.0 && SOR < 3.0 && MQRankSum > −12.5 && ReadPosRankSum > −8.0'). Sequencing data were analysed to identify crossovers using the TIGER analysis pipeline (Rowan *et al.*, 2015) with manual curation. For CNV analysis we first used the bedtools multicov function to generate a bed file containing read depth in 100-kb windows. Coverage in each window was then normalised by average read depth for that window across all individuals. The R package 'SLMSEG' (Orlandini *et al.*, 2017) was then used to characterise CNV.

Orthologue identification

To identify *A. thaliana* orthologues of *A. suecica* genes and homoeologous gene pairs within *A. suecica*, we first extracted the coding sequence (CDS) of the primary transcript of each gene and then performed BLAST searches for each CDS against the sets of CDS from the two non-(sub)genomes of origin, retaining the best hit only. When the two CDS from different (sub)genomes were reciprocal best BLAST hits, the corresponding genes were designated orthologues/homoeologs (Supporting Information Tables S1–S3).

Results

Reduced fertility in synthetic *A. suecica* is associated with an increase in univalents, multivalents, chromosome bridges and lagging chromosomes during meiosis

Synthetic *Arabidopsis suecica* lines showed large reductions in pollen viability, with some variation between different synthetic lines (Fig. S1) as has been observed previously (Chéron *et al.*, 2024). Meiotic progression in natural and synthetic *Arabidopsis suecica* was initially characterised using DAPI-stained meiotic spreads of

developing anthers (Fig. 1). Meiosis in natural and synthetic *A. suecica* progressed similarly in early prophase but diverged by pachytene (Fig. 1a). While pachytene cells with complete synapsis were observed in natural lines, a true pachytene stage was never observed in synthetic *A. suecica*, with all pachytene-like cells retaining some un-synapsed regions (Fig. 1a,b). In some instances, large asynaptic loops were observed (Fig. 1a,b), which could potentially arise from nonhomologous synapsis. At metaphase I, synthetic lines showed a large increase in the number of meiotic errors, with the majority of cells showing multivalents and/or univalents (Fig. 1a,c). Similarly, lagging chromosomes and chromosome bridges were observed in the majority of anaphase I cells in synthetic lines but rarely in natural accessions (Fig. 1a,c). Lagging DAPI-stained bodies were also regularly observed at anaphase II in synthetic lines but not in natural *A. suecica* (Fig. 1a). The exact nature of these bodies is unclear but could represent single chromatids with bipolar kinetochore attachments or acentric chromosome fragments generated via nonhomologous recombination. Finally, extranuclear DAPI-stained bodies were observed in most telophase I cells and tetrads in synthetic *A. suecica*, occasionally accompanied by inter-nuclei bridges, but were rarely observed in natural lines (Fig. 1a,c).

We also estimated crossover number based on chiasma counts, observing significantly fewer crossovers in synthetic plants (Fig. 1d). These results should be taken with some caution as, for many synthetic metaphase I cells with multivalents, we were unable to determine accurate counts and this may have introduced some biases. However, our counts are in good agreement with crossover number determined by MLH1 foci counts in natural and synthetic *A. suecica* by Chéron *et al.* (2024).

Fluorescent *in situ* hybridisation using subgenome-specific centromeric probes reveals nonhomologous recombination in synthetic *A. suecica*

To further investigate the nature of the meiotic defects observed in synthetic *A. suecica*, we performed FISH using probes able to distinguish the centromeric repeat sequences of the two subgenomes. At metaphase I in natural *A. suecica*, as observed previously (Nibau *et al.*, 2022), we observed no multivalents and bivalents always comprised of two chromosomes labelled with the same probe (i.e. both chromosomes were from the same subgenome). By contrast, at metaphase I over 50% of cells ($n = 19$) in synthetic *A. suecica* showed clear evidence of nonhomologous crossover formation, with bivalents or multivalents comprising of chromosomes hybridising to both probes (Fig. 2).

Synthetic *A. suecica* has incomplete synapsis

We used immunolabelling of ASY1, a component of the chromosome axis (Armstrong *et al.*, 2002), and ZYP1, the transverse filament of the synaptonemal complex (Higgins *et al.*, 2005), to monitor the progression of synapsis in *A. suecica* by confocal microscopy. In both natural and synthetic *A. suecica*, we observed discrete ASY1 foci (Fig. 3) which progressed to form linear chromosome axes by early zygotene (Fig. 3). In natural lines, synapsis progressed until maximal synapsis was observed at pachytene, with small regions of strong ASY1 signal remaining, likely corresponding to un-synapsed rDNA repeats (Hurel *et al.*, 2018; Sims *et al.*, 2019). By contrast, we never observed a true pachytene stage with complete synapsis in synthetic *A. suecica* (Fig. 3), consistent with our observations from DAPI chromosome spreads (Fig. 1). Although we never observed complete synapsis ($n = 25$), it is possible that a true pachytene stage was achieved in some cells at low frequency.

Synthetic plants give rise to higher rates of aneuploidy and homoeologous exchange in progeny

Given the meiotic errors observed cytologically in synthetic lines, we anticipated that there was considerable nonhomologous synapsis and crossover formation occurring (presumably between homoeologs). Nonhomologous (including homoeologous) crossovers generate chimeric chromosomes which, when transmitted to progeny, result in reciprocal CNVs in the two chromosomes involved. When such events occur between syntenic homoeologs they are referred to as homoeologous exchanges (e.g. He *et al.*, 2016; Lloyd *et al.*, 2018). To identify and quantify such events arising from meiosis in synthetic and natural lines, we re-sequenced 67 F1 progeny generated by crossing natural and synthetic *A. suecica* as well as 28 progeny from self-fertilised natural *A. suecica*. The primary reason for sequencing F1 individuals was that our synthetic lines were not self-fertile and so no selfed progeny were obtained. When reciprocal crosses were performed to generate the F1 progeny, crosses had much less success when the synthetic parent was the pollen donor compared with crosses where the natural parent was the pollen donor (0 vs 3.8 seeds per cross; $P < 0.05$, *t*-test), possibly reflecting a particular vulnerability in the male germline in adapting to polyploidy (Westermann *et al.*, 2024), and a possible explanation for the lack of selfed seeds obtained from synthetic plants.

Reciprocal CNVs and other structural variants, for example aneuploidy or large insertions or deletions (INDELs), were identified by running segmentation analysis (Orlandini *et al.*, 2017) on normalised read depth across the genome (Fig. 4a). Sequenced individuals were then genotyped using the TIGER (Rowan *et al.*, 2015) so that CNVs could be unambiguously attributed to the parent of origin (i.e. synthetic or natural) based on polymorphism between the natural and synthetic lines (Fig. S2). All reciprocal CNVs we observed occurred between homoeologs within syntenic blocks (Fig. 4b). This indicates that nonhomologous crossovers in *A. suecica* occur primarily (and likely exclusively) between homoeologs. To our surprise, we observed a large number of homoeologous exchanges originating from the natural parent in both F1 individuals and progeny of self-fertilised natural plants (Tables S4, S5). On closer inspection, it became clear that in almost every case (42/44), the homoeologous exchanges inherited from natural parent had identical coordinates and thus were clearly pre-existing events segregating in our natural lines. Two accessions of natural *A. suecica* were used in our study: the reference line AsS3 (Burns *et al.*, 2021) and our standard laboratory strain JC2, derived from reference strain As9502 (Jiang

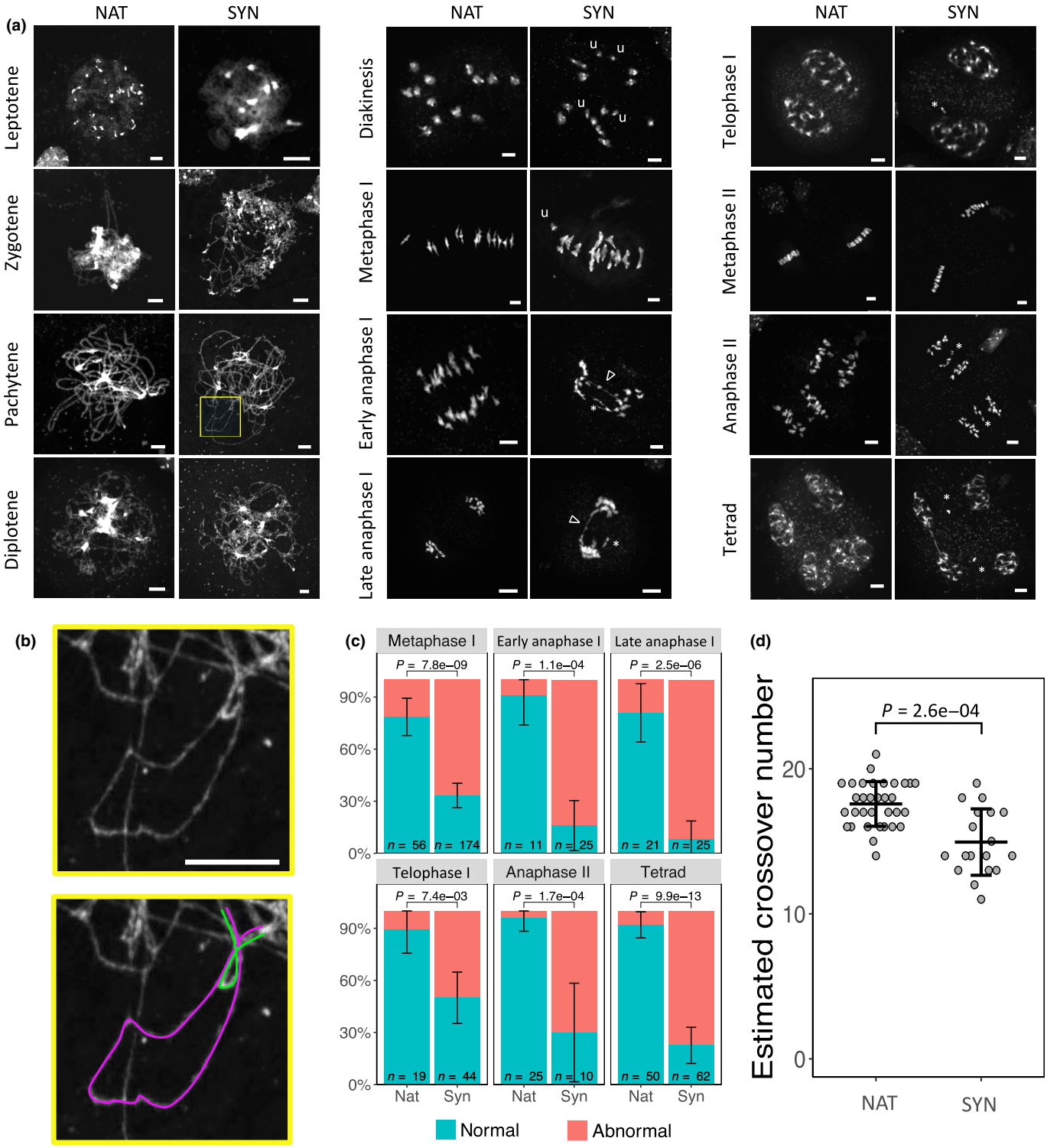

Fig. 1 Meiotic progression in natural and synthetic *Arabidopsis suecica*. (a) DAPI-stained meiotic cell nuclei. In synthetic plants, errors are frequently observed including: univalents at metaphase I (u); chromosome bridges (arrowheads) and lagging chromatin (*) at anaphase I; lagging/extranuclear chromatin in telophase I, anaphase II and tetrads. (b) Magnification of an asynaptic loop in the pachytene-like cell in synthetic *A. suecica* in A (yellow square). Bars: (a, b) 5 μm. (c) Quantification of meiotic errors in natural and synthetic lines. Significantly, more errors are observed in synthetic *A. suecica* in all stages from metaphase I, chi-squared test. Error bars show 95% confidence interval. (d) Estimated crossover number based on chiasma counts in natural and synthetic metaphase I cells. Significantly, fewer crossovers are observed at metaphase I in synthetic plants, Mann–Whitney *U*-test. Mean (horizontal line) and SD (error bars) are shown. NAT, natural; SYN, synthetic.

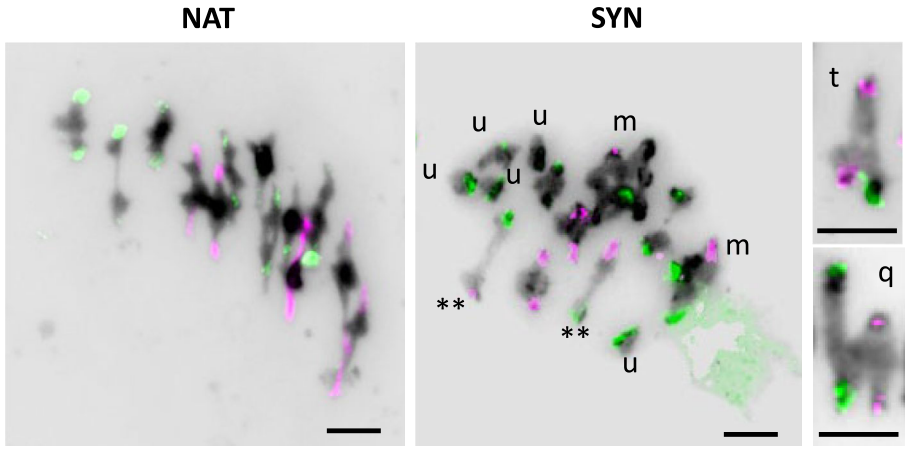

Fig. 2 Fluorescence *in situ* hybridisation (FISH) of centromeric repeats in natural and synthetic *Arabidopsis suecica* meiocytes. Probes specific to the *Arabidopsis arenosa* (green) and *Arabidopsis thaliana* (pink) centromeric repeat sequences were hybridised to *A. suecica* meiocytes. At metaphase I in natural (NAT) *A. suecica*, the two chromosomes in each bivalent were always labelled by the same coloured probe and multivalents were not observed. In synthetic (SYN) plants, the two centromeres of some bivalents were labelled by different coloured probes indicating nonhomologous crossover formation (**). Clear trivalents (t), quadrivalents (q) and more complex multivalents (m) involving both *A. thaliana* and *A. arenosa* subgenome chromosomes were also observed, as were univalents (u). Bars, 5 μm.

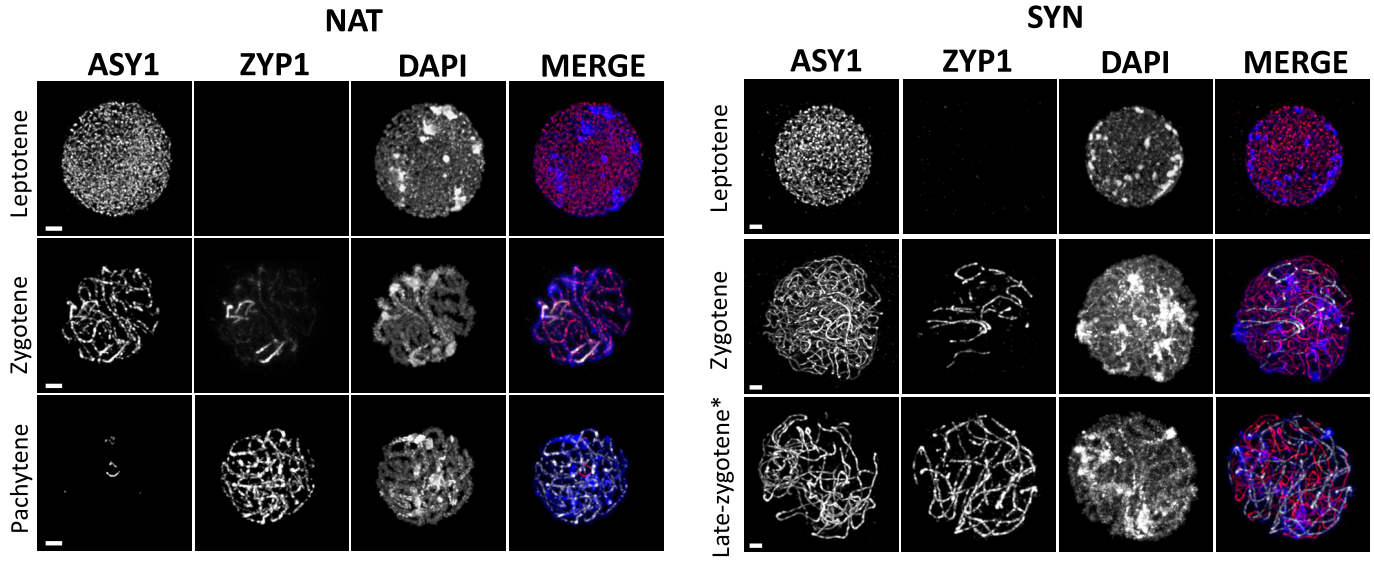

Fig. 3 Progression of synapsis in natural and synthetic *Arabidopsis suecica*. 3D-confocal images showing localisation of ASY1 (red), ZYP1 (grey) and DNA (DAPI, blue) in early meiotic prophase I. ASY1 first appears as distinct foci at leptotene, progressing to fully extended axis by zygotene. Synapsis begins in zygotene with maximal synapsis achieved at pachytene. * In synthetic *A. suecica*, a true pachytene stage cell with full synapsis was never observed. Bars, 5 μm. NAT, natural; SYN, synthetic.

et al., 2021; Nibau *et al.*, 2022). Homoeologous exchanges originating from the natural parent were observed in most selfed (16/17) and F1 (15/28) progeny derived from AsS3 but never from selfed (0/11) or F1 (0/38) progeny derived from JC2, indicating that they were segregating within our AsS3 material.

In contrast to homoeologous exchanges originating from the natural parent, all homoeologous exchanges originating from the synthetic parent had unique coordinates (17/17) and thus arose *de novo* in the meioses that gave rise to the F1 individuals. The rate of *de novo* homoeologous exchange originating from meiosis in the synthetic parent was over 20-fold higher than those originating from meiosis in natural lines (16.4% vs 0.8% respectively; $P < 0.0001$; Fisher exact test).

In addition to homoeologous exchanges, our CNV analysis also identified cases of aneuploidy. Aneuploidy originating from synthetic gametes (e.g. Fig. 4; SYN X NAT–54) was over eight-fold higher than aneuploidy originating from natural gametes (13.6% vs 1.6% respectively; $P = 0.0016$; Fisher exact test).

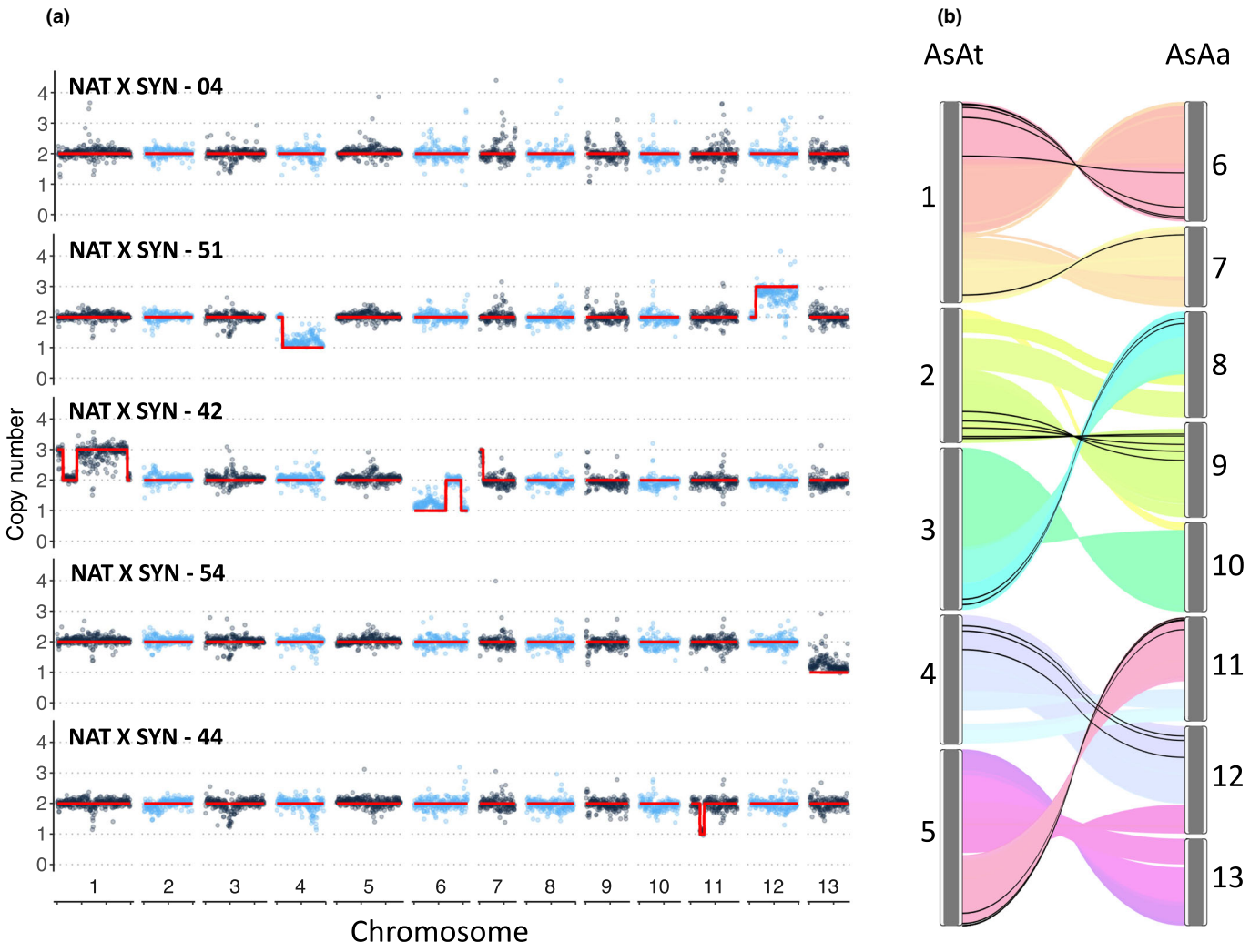

Fig. 4 Copy number variation in progeny of synthetic and natural *Arabidopsis suecica*. (a) Read depth segmentation analysis using whole-genome re-sequencing data of selected F1 progeny derived by crossing synthetic and natural *A. suecica*. Dots indicate normalised read depth in 100 kb genomic windows. Output of the segmentation analysis is plotted as red lines indicating predicted copy number. Reciprocal copy number changes in syntenic regions of the *Arabidopsis thaliana* subgenome (Chromosomes 1–5) and *Arabidopsis arenosa* subgenome (Chromosomes 6–13) are diagnostic of homoeologous crossover formation (e.g. SYN X NAT-48, SYN X NAT-39). Whole chromosome aneuploidy (e.g. SYN X NAT-54) and nonreciprocal INDELs (e.g. SYN X NAT-44) were also identified. (b) Synteny plot of the *A. thaliana* and *A. arenosa* subgenomes of *A. suecica*, showing the location of *de novo* homoeologous exchanges (black lines) identified. Coloured segments indicate syntenic blocks. NAT, natural; SYN, synthetic.

Aneuploidy occurred in both the *A. thaliana* (3/11) and *A. arenosa* (8/11) subgenomes and led to both chromosome additions ($2n = 27$; 4/11) and losses ($2n = 25$; 7/11).

Homoeologous exchanges are enriched in sub-telomeric regions and occur preferentially within genes

Homoeologous exchanges generate chimeric chromosomes with sequence originating from both *A. thaliana* and *A. arenosa* subgenomes. Based on the extensive polymorphism between the two subgenomes, we were in most cases able to identify paired-end sequencing reads that overlapped homoeologous exchange junctions, enabling us to fine map the exchange positions to precise genomic windows (Fig. 5a; median 67 bp, mean 146 bp). Homoeologous exchanges occurred, on average, closer to the nearest telomere than the same number of genomic positions taken at random (*c.* 2.8 Mb vs *c.* 5.1 Mb; $P < 0.01$, Wilcoxon rank sum test; Fig. 5b). To see whether this could be explained by homoeologous exchanges occurring with a similar distribution to that of homologous crossovers, we also compared the positions of homoeologous exchanges to the same number of positions generated from genome-wide crossover distributions. For this comparison, as there are no published genome-wide recombination data for *A. suecica*, genomic positions were generated according to *A. thaliana* male and female crossover distributions (Basu-roy *et al.*, 2013). Homoeologous exchanges occurred, on average, closer to the nearest telomere than the same number of positions generated from either male or female crossover distributions (2.5 Mb for HEs vs 5.2 Mb for male COs, $P < 0.05$, and 7.7 Mb for female COs, $P < 1.2e{-}05$, Wilcoxon rank sum

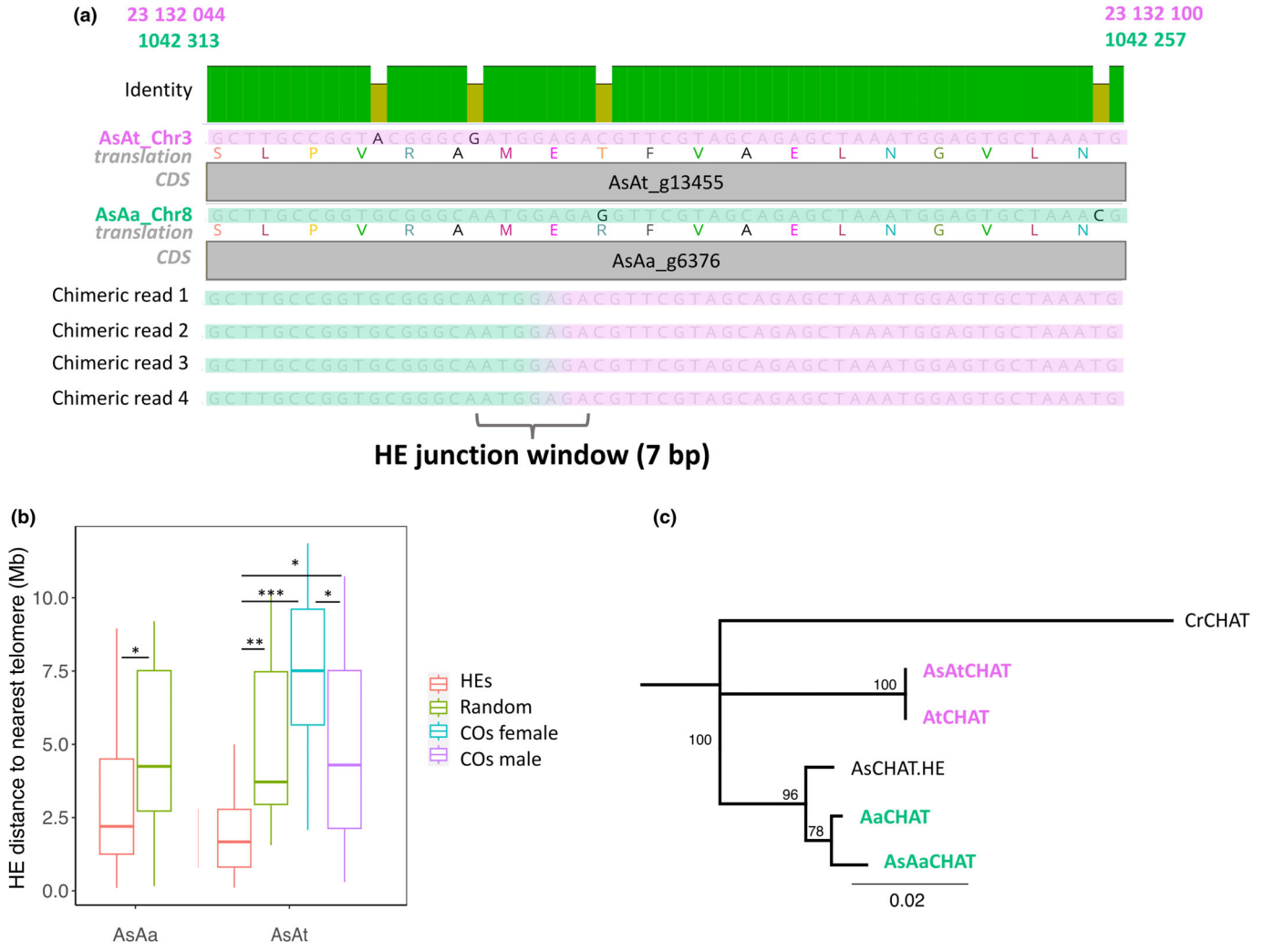

Fig. 5 Homoeologous exchanges are distally located and regularly generate chimeric genes encoding novel protein variants. (a) Example fine mapping of a homoeologous exchange (HE) junction window. Identification of chimeric reads flanking a homoeologous exchange in individual NAT_X_SYN_016 enabled the junction to be fine mapped to a 7 bp window. (b) Homoeologous exchange points were, on average, found closer to telomeres than random genomic positions taken from either a uniform distribution (random), or from genome-wide crossover distributions from female or male *A. thaliana* (COs female and COs male, respectively). Boxes indicate 25th and 75th percentiles, whiskers indicate 1.5× IQR (IQR: the distance between the first and third quartiles). (c) Phylogeny based on acetyl CoA:(Z)-3-hexen-1-ol acetyltransferase (CHAT) protein sequence; a homoeologous exchange in individual NAT_X_SYN_016 generated a chimeric gene encoding a new variant of this protein (AsCHAT.HE), which branches as an outgroup to AaCHAT/AsAaCHAT. Species codes for phylogeny: Aa, *A. arenosa*; AsAa, *A. arenosa* subgenome of *A. suecica*; At, *A. thaliana*; AsAt, *A. thaliana* subgenome of *A. suecica*; Cr, *Capsella rubella*. Branch lengths represent estimated amino acid substitutions per site. Significance codes for box plot, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$, ANOVA with *post hoc* pair-wise *t*-tests and Bonferroni correction.

test; Fig. 5b). The distal localisation of homoeologous exchanges is particularly striking given that (a) most events in our analysis were known to have occurred in the female germline (16/19) and (b) that homologous crossovers show a strong proximal bias in female *A. thaliana* meiosis (Basu-roy *et al.*, 2013).

In addition to their distal location, most homoeologous exchanges that we could precisely locate, occurred between gene start and stop codons (12/18), significantly more than expected by chance ($P = 0.015$; chi-squared test). Such exchanges generate new chimeric versions of genes, containing sequence derived from both the *A. thaliana* and *A. arenosa* subgenomes and can thus encode novel protein variants (Fig. 5c; Table S5). We wondered whether the preference for homoeologous exchanges to occur within genes might reflect their greater sequence conservation compared with intergenic regions. To address this question, we determined the sequence identity between homoeologs in the 1-kb region surrounding the midpoint of the homoeologous exchange junction windows and compared this with the same measure determined from random genomic windows. We observed a localised peak in sequence identity in the 100-bp window centred on the midpoint of homoeologous exchange junction (Fig. S3), though given the number of events we were able to analyse, confidence intervals were large, and the difference was not significant ($P = 0.077$, Wilcoxon rank sum test).

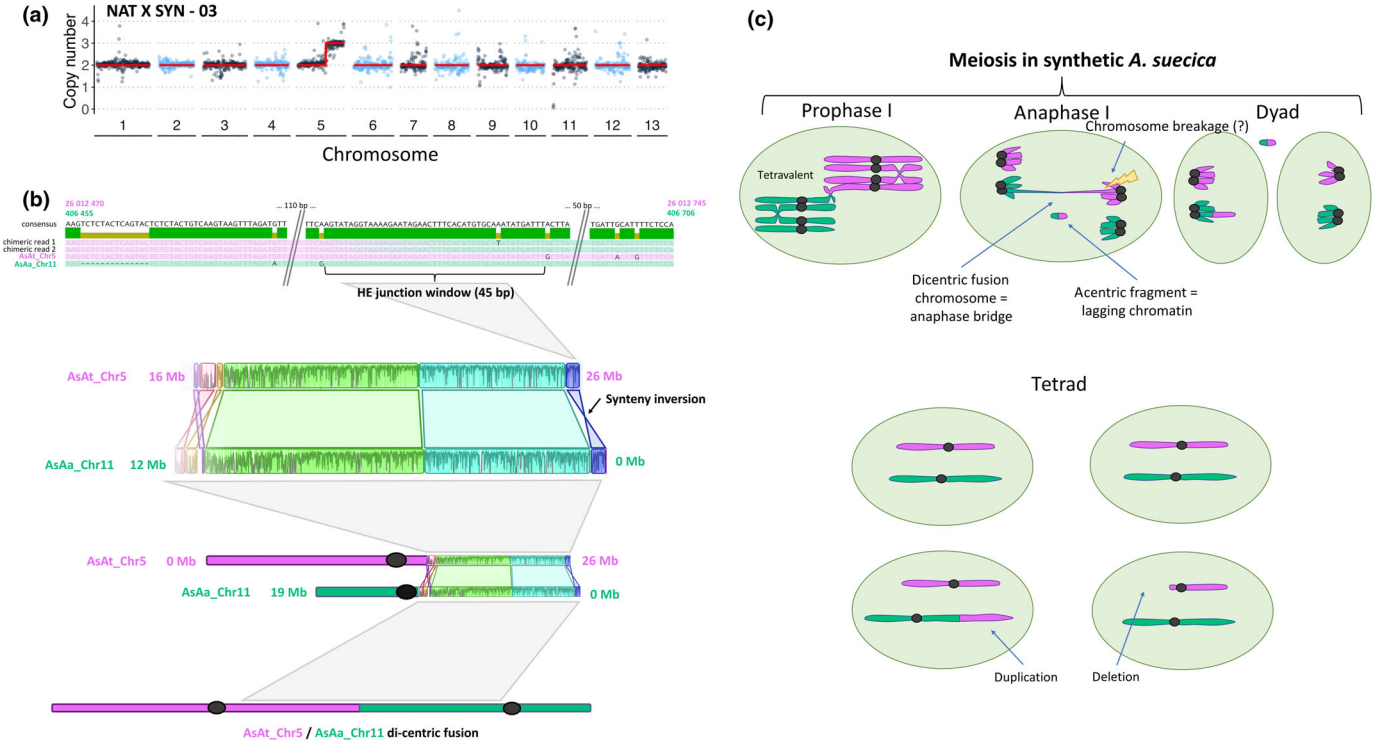

Fig. 6 Homoeologous exchanges in syntenic inversions generate large duplications and explain observed cytological phenotypes. (a) Read depth segmentation analysis for individual NAT X SYN-03 showed a large duplication of a region of Chromosome 5. Dots indicate normalised read depth in 100 kb genomic windows. Red lines indicate predicted copy number. (b) Chimeric reads were identified mapping to syntenic regions on Chromosomes 5 and 11, indicating that the duplication is associated with homoeologous crossover formation. Whole chromosome alignment revealed that the site of homoeologous crossover formation is within a sub-telomeric syntenic inversion such that the homoeologous crossover would have generated a dicentric fusion chromosome. (c) A model for how homoeologous crossover formation in syntenic inversions can explain the copy number variant (CNV) observed in individual NAT X SYN-03 and many of the observed cytological phenotypes in synthetic *Arabidopsis suecica*. NAT, natural; SYN, synthetic.

Homoeologous exchange within syntenic inversion results in nonreciprocal CNV

While most homoeologous exchanges resulted in obvious reciprocal changes in copy number (Fig. 4), this was not true in every case. When analysing the junction of a large-scale duplication (i.e. a *non*reciprocal CNV; Fig. 6a) we identified chimeric reads which on one side matched Chromosome 5 (*A. thaliana* subgenome) and on the other matched the syntenic region on Chromosome 11 (*A. arenosa* subgenome), indicating that the duplication arose via homoeologous exchange. Whole chromosome alignment indicated that the homoeologous crossover between the end of Chromosome 5 and the start of Chromosome 11 occurred within a local syntenic inversion (Fig. 6b). Such an event would have resulted in the generation of a dicentric fusion chromosome (Fig. 6b,c). The duplication spanned the region beginning at the site of the homoeologous exchange and ended adjacent to the centromere on Chromosome 5. One way the duplication could have arisen is via chromosome breakage associated with bipolar segregation of the two centromeres on the single chromosome (Fig. 6c). Such events would likely result in bridges and lagging chromatin at anaphase I (Fig. 6c) and could, at least in part, explain many of the observed cytological phenotypes in synthetic lines (e.g. Fig. 1).

Discussion

Polyploid plants are widespread in nature but face numerous hurdles becoming established. Perhaps the most widely acknowledged hurdle is that of ensuring faithful meiotic recombination and segregation with an additional set of chromosomes. While there have been recent advances in our understanding of meiotic adaptation in autopolyploids (Yant *et al.*, 2013; Seear *et al.*, 2020; Morgan *et al.*, 2021, 2022; Gonzalo, 2022), we still know relatively little about this process in allopolyploid plants. We present here a systematic comparison of meiosis in naturally evolved and first-generation synthetic lines of the model allopolyploid *A. suecica*, highlighting the extensive meiotic errors, and their consequences, in synthetic lines.

We have previously demonstrated that meiosis in established *A. suecica* is largely diploid-like, with normal synaptic progression and no evidence of crossover formation between homoeologous chromosomes (Nibau *et al.*, 2022). While we did observe some low-frequency errors including univalents at metaphase I and anaphase bridges (Nibau *et al.*, 2022), these had minimal impact on fertility, with natural accessions having high pollen viability and seed set. By contrast, in our current study we observed very low fertility and frequent meiotic errors in first-generation synthetic *A. suecica*. These errors, including asynapsis and

homoeologous crossover formation, are characteristic of new allopolyploids (Szadkowski *et al.*, 2010) and confirm *A. suecica* as an important model allopolyploid system for meiosis research.

Meiotic errors became apparent in early prophase with the failure to observe fully synapsed pachytene cells in synthetic lines. Asynapsis is a common feature of meiosis in first-generation allopolyploids and is also seen in wheat *ph1* mutants and 5B nullisomics (Holm & Wang, 1988), which only achieve *c.* 40% synapsis by the end of pachytene, suggesting that ensuring correct synaptic progression is the primary hurdle facing new allopolyploids. Our favoured hypothesis is that incomplete synapsis is an indirect effect of failures in accurate chromosome pairing in early prophase. Though further experiments will be required to confirm this, we did see cytological evidence of nonhomologous synapsis and crossover formation in our study, including asymmetric, asynaptic loops in pachytene-like cells (Fig. 1b) and nonhomologous bivalents at metaphase I (Fig. 2). There are many examples of nonhomologous synapsis and crossover formation in the existing literature, for example synapsis and bivalents in allohaploids (Holm & Wang, 1988; Grandont *et al.*, 2014) or interspecific hybrids (Holm & Wang, 1988; Martin *et al.*, 2017; Rey *et al.*, 2021) where true homologues are absent. In general, it is presumed that all, or at least the vast majority of, nonhomologous synapsis and crossover formation in allopolyploids or interspecific hybrids occurs between related homoeologs. However, explicitly demonstrating this cytologically is nontrivial, given the need to specifically label a single pair of homoeologous chromosomes. Despite the lack of direct cytological evidence, our re-sequencing data of progeny of synthetic *A. suecica* plants clearly demonstrate that the vast majority of (and possibly all) nonhomologous exchanges that make it through to the next generation, occur between homoeologs. We cannot, however, rule out the possibility that some crossovers do occur between completely unrelated chromosomes, but that the resulting genomic configurations are not tolerated and are selected against. Regardless, the prevalence of homoeologous exchanges that we do observe implies a high degree of homoeologous synapsis at the time of crossover maturation (late pachytene) in first-generation *A. suecica*. Given the large degree of structural divergence (five chromosomes vs eight chromosomes) and primary sequence divergence (*c.* 85–90% nucleotide identity) between the two subgenomes, the extent of homoeologous crossover formation in synthetic lines is quite striking and highlights the fact that meiotic recombination is able to occur between sequences with considerable levels of divergence. This is in contrast to somatic homologous recombination in Arabidopsis, which is largely abolished with only 4% divergence between recombining sequences (Li *et al.*, 2009). In diploid organisms, some accommodation of divergence during meiosis is likely useful, for example to facilitate crossovers between homologous chromosomes harbouring different haplotypes. This is particularly true in outcrossing species such as *A. arenosa* where homologous chromosomes regularly have *c.* 3% divergence (Monnahan *et al.*, 2019). In allopolyploids, however, this comes at a cost and more stringent crossover formation, such as that shown by natural lines of *A. suecica*, is required to prevent homoeologous crossovers. It is also worth noting that meiosis is still not perfect in natural *A. suecica* with both aneuploidy and homoeologous exchange occurring at low frequency, as is observed in other established allopolyploids of recent origin (e.g. Lloyd *et al.*, 2018).

Homoeologous synapsis in synthetic allopolyploids must arise from either the failure to correct, or to prevent, homoeologous synaptic associations. In wild-type hexaploid wheat, it appears to be the former as nonhomologous synaptic associations are observed in zygotene, but by early pachytene only bivalent associations persist (Hobolth, 1981). This synaptic correction, however, does not occur in haploid wheat, wheat-rye hybrids or haploid *Brassica napus*. In these instances, chromosomes have homoeologs but no homologues and extensive nonhomologous (presumably homoeologous) synapsis is observed (up to 90% in some genotypes), even in the presence of pairing control loci (Holm & Wang, 1988; Grandont *et al.*, 2014). Correction of nonhomologous synapsis may therefore require the presence of the true homologue, suggesting competition between potential synaptic partners. In a recent study, Chéron *et al.* (2024) observed higher levels of inter-subgenomic centromeric association during zygotene in synthetic compared with natural *A. suecica*, suggesting that the dynamics of early chromosome paring and synaptic initiation are important for ensuring high-fidelity synapsis between homologues and suppressing homoeologous crossover formation.

Another striking phenotype we regularly observed in synthetic *A. suecica* was lagging chromatids at anaphase II. Similar phenotypes have been described in Arabidopsis *scc3* (Chelysheva *et al.*, 2005), *apc8* (Xu *et al.*, 2019) and *smg7* (Riehs *et al.*, 2008) mutants and have been ascribed to bipolar kinetochore attachment (Chelysheva *et al.*, 2005), or spindle disorganisation (Xu *et al.*, 2019). Similar defects may underlie the lagging chromatids observed at anaphase II in synthetic *A. suecica* and could indicate aspects of neo-allopolyploid meiosis, unrelated to homoeologous synapsis and crossover formation that also require adaptation, for example kinetochore structure.

Our whole-genome re-sequencing of progeny of synthetic and natural plants provided further insights into the meiotic errors observed in synthetic *A. suecica* through analysis of the structural rearrangements fine mapped in progeny. All reciprocal changes in copy number (which indicate nonhomologous crossover formation) occurred within syntenic blocks on homoeologs and were biased towards distal ends of chromosomes, something that has also been observed in resynthesised tetraploid wheat (Zhang *et al.*, 2020). In *A. suecica* at least, the proximity of these events to the telomere is not just a simple reflection of a distal bias in crossover formation generally, as homoeologous exchanges also showed a strong distal bias relative to the distribution of homologous crossovers. This bias was particularly striking as most events we characterised occurred via the female germline and homologous crossovers in female Arabidopsis meiosis are proximally distributed (Basu-roy *et al.*, 2013; Lloyd & Jenczewski, 2019). One possible explanation is that homoeologous crossovers in proximal regions lead to larger structural rearrangements which are less tolerated and therefore selected against in gametes and/or during embryo development. Alternatively, it is possible that

homoeologous crossovers occur more frequently in distal regions than homologous crossovers. Such a scenario could be envisaged if, for example, homoeologous synapsis primarily occurred in distal regions. The distal bias of homoeologous crossovers has implications for trait introgression in plant (pre)-breeding programmes and suggests that the already challenging introgression of traits associated with genes in interstitial or proximal regions may be even more challenging in wider crosses, for example with wild-crop relatives.

As was observed in synthetic wheat (Zhang *et al.*, 2020), homoeologous exchanges in synthetic *A. suecica* occurred predominantly within genes. These exchanges regularly generate new chimeric versions of genes with sequence from both subgenomes that are predicted to encode novel protein variants. Given the large structural rearrangements often generated by homoeologous exchanges, these new variants may be regularly selected against. Despite this, some chimeric genes associated with homoeologous exchange do persist within established populations (Zhang *et al.*, 2020) and could potentially be adaptive, as has been shown for interspecific chimeric alleles observed in *A. arenosa*/*A. lyrata* hybrid zones (Seear *et al.*, 2020).

The cause of the preference for homoeologous exchanges to occur within genes is unclear, particularly given that in Arabidopsis, meiotic double-strand breaks (the precursors of crossovers) are highest in promoters and terminators and lower in gene bodies (Choi *et al.*, 2018). One possibility is that the bias for homoeologous exchanges to occur within genes relates to the higher sequence conservation associated with CDS compared with intergenic regions.

While in most cases, homoeologous exchanges do not provide any structural barrier to chromosome segregation, problems do arise when crossovers occur within regions where synteny between the homoeologs has been inverted (Fig. 6). It has long been known that inversions pose a major barrier to transmission of single recombinants (Sturtevant & Beadle, 1936). Such events generate dicentric chromosomes which will form anaphase I bridges (such as those seen in Fig. 1a) if the two centromeres are pulled towards opposing poles of the dividing cell (Fig. 6b,c). They will also generate acentric chromosome fragments and likely contribute to the lagging and/or extranuclear chromatin bodies we frequently observed in meiocytes of synthetic plants (Fig. 1a). We show that despite the barriers, these events are occasionally transmitted to progeny and can generate large megabase scale INDELs, presumably due to breakage of the dicentric chromosome during the first meiotic division.

The observation that crosses involving a synthetic female parent were more successful than those involving a synthetic male parent are intriguing and suggest adaptation to polyploidy is more challenging for male reproduction. One possible explanation is that the lower recombination rate in female Arabidopsis meiosis (Lloyd & Jenczewski, 2019) means that there is less opportunity for illegitimate crossover formation. Alternatively, it is possible that the difference stems from postmeiotic challenges. For example, Westermann *et al.* (2024) recently demonstrated that pollen tube tip growth is severely challenged in first-generation autopolyploid *A. thaliana* and *A. arenosa*. A similar defect in first-generation allopolyploid *A. suecica* could also explain the reduced success in crosses using synthetic *A. suecica* as the pollen donor. *Arabidopsis suecica* provides a highly tractable system for forward genetic (e.g. mutagenesis screens and mapping populations) and reverse genetics (e.g. CRISPR mutagenesis) approaches. Such experiments will be important for developing a systematic understanding of the reproductive challenges faced by new allopolyploids and how they are overcome. Further insights are also likely to be gained from an in-depth study of the early steps of chromosome pairing and synapsis. Such analyses, if combined with tools such as oligo paints (Nguyen & Joyce, 2019) to differentially label homologues and homoeologs, will be particularly powerful and provide important insights into how crossover stringency is determined.

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Competing interests

None declared.

Author contributions

CN and AL designed the research. CN, AE, HK and AL performed the research. CN, DWP and AL analysed data. AL wrote the paper with input from all authors.

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Data availability

Short-read sequencing data are available under the NCBI BioProject PRJNA1103975.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Pollen viability.

Fig. S2 Attribution of CNVs to parent of origin.

Fig. S3 Identity of HE junction flanking sequence.

Table S1 CDS reciprocal best BLAST hits: AsAa–AsAt.

Table S2 CDS reciprocal best BLAST hits: AsAa–At.

Table S3 CDS reciprocal best BLAST hits: AsAt–At.

Table S4 CNVs by individual.

Table S5 HEs by event.

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